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(54) Thermostable human Cu/Zn superoxide dismutase muteins.

(57) Thermostable muteins of human Cu/Zn superoxide dismutase (hSOD) in which one or both of the free cysteine residues at positions 6 and 111 are replaced with an uncharged amino acid. hSOD Ala6, hSOD Ser111, and hSOD Ala6 Ser111 are specifically exemplified.

EP 0 275 202 A2

D scription

THERMOSTABLE HUMAN Cu/Zn SUPEROXIDE DISMUTASE MUTEINS

Description

Technical Field

This invention is in the fields of protein chemistry and genetic engineering. More particularly it relates to muteins of human Cu/Zn superoxide dismutase in which either or both of the free cysteines at amino acid positions 6 and 111 of the native protein have been replaced with a neutral amino acid.

Background

Superoxide dismutases are a family of enzymatic proteins that include associated metal ions. The members of the family differ in both amino acid sequence and in the type of associated metals. Their ability to catalyze the destruction of superoxide ions renders them useful as pharmaceuticals, in cosmetics, and in food preservation. The amino acid sequence of human Cu/Zn superoxide dismutase (hereinafter referred to as hSOD) is described in Jabusch et al, Biochemistry (1980) 19:2310-2316. The cloning and sequencing of hSOD cDNA and the production of hSOD in bacteria and yeast are described in EPA 84111416.8 (published 24 April 1985 under number 0 138 111).

One potential shortcoming of hSOD is its relative lability to heat. This property limits its shelf life and its ability to be made into pharmaceutical, food, or cosmetic formulations that may be subjected to elevated temperatures. In this regard, it is known that yeast Cu/Zn SOD contains no free cysteines, but is less thermostable than bovine SOD or hSOD (Steinman, H. M. in Superoxide Dismutase Vol I, pp 18-19, CRC Press, 1982). The only reference relating to increasing the thermal stability of hSOD is Jabusch et al, supra. It reports that alkylation of Cys 111 of native hSOD with iodoacetate yields a more stable molecule.

The only reference that describes a mutein of hSOD is Hallewell et al, Nucleic Acids Research (1985) Vol 13, No 6, pp 2017-2034. It reports a hSOD mutein having an Ala → Glu substitution at position four. No dismutation activity was detected for that mutein and it was thus estimated that its activity was reduced more than 20-fold relative to the native molecule.

Cysteine modifications have been made in other molecules. For instance, U.S. Pat No. 4,518,584 describes muteins of lymphokines in which cysteines that are not essential for biological activity are replaced with a neutral amino acid to facilitate the production of molecules with proper disulfide bridging in bacteria. Perry and Wetzel, Science (1984) 226:555-557 describe a mutein of T4 lysozyme having an Ile → Cys substitution at position three, a disulfide link between positions 3 and 97, and a free cysteine at position 54. Alkylation of that free cysteine increased the thermal stability of the mutein.

In sum the art suggests that alkylation of Cys 111 of native hSOD increases thermal stability, but is silent as to whether an amino acid substitution at position 111 or other positions would result in a functional molecule and whether such a change would increase the thermostability of the molecule. Indeed, the only reported mutein of hSOD lacked dismutase activity and the relative thermostabilities of yeast, bovine and human SODs appear to indicate that thermostability is dependent upon something other than the free cysteine content of the molecule.

Disclosure of the Invention

Applicants have found that the substitution of either or both of the free cysteines at positions 6 and 111 of hSOD with an uncharged (neutral) amino acid enhances the thermal stability of the molecule. hSOD with both of these cysteines replaced is significantly more thermostable than hSOD with only a single cysteine replaced. Hence, hSOD with both cysteines replaced is preferred.

Accordingly, muteins of hSOD in which at least one of the cysteine residues at positions 6 and 111 is replaced with an uncharged amino acid are one aspect of the invention.

Another aspect of the invention is a method of enhancing the thermostability of hSOD comprising replacing at least one of the cysteine residues at positions 6 and 111 of hSOD with an uncharged amino acid.

Pharmaceutical and cosmetic compositions containing such muteins are yet another aspect of the invention.

The DNA, expression vectors and recombinant organisms that are used to make the above described muteins are also part of the invention.

Brief Description of the Drawings

Figure 1 shows the sequence of a DNA fragment that encodes hSOD and the amino acid sequence of hSOD.

Figure 2 is a flow chart for the construction of plasmid pYSODA, a yeast expression plasmid used to produce a mutein of hSOD having a Cys → Ala substitution at position 6 of the amino acid sequence shown in Figure 1 (the mutein is designated hSOD Ala6).

Figure 3 is a flow chart for the construction of plasmid pSODCF1, an intermediate used in the construction of plasmid pYSODAS described below.

Figure 4 is a flow chart for the construction of the mutant M13 clone M13rp8SODCIIIS, an intermediate construct used to prepare plasmid pYSODS described below.

Figure 5 is a flow chart for the construction of plasmid pYSODS, a yeast xpression plasmid used to produce a mutein of hSOD having a Cys → Ser substitution at amino acid position III of th s quence shown in Figure I (the mutein is designated hSOD SerIII).

Figure 6 is a flow chart for the construction of plasmid pYSODAS, a yeast expression plasmid for producing a mutein of hSOD having a Cys → Ala substitution at position 6 and a Cys → Ser substitution at position III (the mutein is designated hSOD Ala6 SerIII).

Figures 7-9 are photographs of various electrophoretic gel analyses described in the examples.

Figures I0-I2 are graphs depicting the relative thermal stabilities of hSOD and the hSOD muteins described in the examples.

Modes for Carrying Out the Invention

The hSOD muteins of the invention have at least one of the two free cysteine residues at positions 6 and III (amino acid numbering herein refers to that of the native molecule shown in Figure I) replaced with an uncharged (nonpolar or uncharged polar), preferably acyclic (nonaromatic, nonheterocyclic) amino acid. Amino acids having hydrogen (i.e., glycine), an aliphatic, or a hydroxylic side chain are particularly preferred replacements. When both cysteines are replaced, the two replacement amino acids may be the same or different. Examples of amino acids that may be used to replace the cysteines are glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, methionine, proline, tryptophan, and tyrosine. Serine and alanine are preferred replacement residues. Other amino acids of the native hSOD sequence may be replaced or deleted provided that such alterations neither affect adversely the dismutase activity of the mutein nor its thermostability. The number of such alterations will usually be less than about 5.

Specific examples of muteins of the invention are hSOD Ala6, hSOD Gly6, hSOD Val6, hSOD Leu6, hSOD Ile6, hSOD Thr6, hSOD Ser6, hSOD Gln6, hSOD Asn6, hSOD Met6, hSOD SerIII, hSOD AlaIII, hSOD GlyIII, hSOD ValIII, hSOD LeuIII, hSOD IleIII, hSOD ThrIII, hSOD GlnIII, hSOD AsnIII, hSOD MetIII, hSOD Gly6 GlyIII, hSOD Ala6 AlaIII, hSOD Leu6 LeuIII, hSOD Ile6 IleIII, hSOD Ser6 SerIII, hSOD Thr6 ThrIII, hSOD Gln6 GlnIII, hSOD Asn6 AsnIII, hSOD Met6 MetIII, hSOD Gly6 AlaIII, hSOD Gly6 ValIII, hSOD Gly6 LeuIII, hSOD Gly6 IleIII, hSOD Gly6 SerIII, hSOD Gly6 ThrIII, hSOD Gly6 GlnIII, hSOD Gly6 AsnIII, hSOD Gly6 MetIII, hSOD Ala6 GlyIII, hSOD Ala6 ValIII, hSOD Ala6 LeuIII, hSOD Ala6 IleIII, hSOD Ala6 SerIII, hSOD Ala6 ThrIII, hSOD Ala6 GlnIII, hSOD Ala6 AsnIII, hSOD Ala6 MetIII, hSOD Val6 GlyIII, hSOD Val6 AlaIII, hSOD Val6 LeuIII, hSOD Val6 IleIII, hSOD Val6 SerIII, hSOD Val6 ThrIII, hSOD Val6 GlnIII, hSOD Val6 AsnIII, hSOD Val6 MetIII, hSOD Leu6 GlyIII, hSOD Leu6 AlaIII, hSOD Leu6 ValIII, hSOD Leu6 IleIII, hSOD Leu6 SerIII, hSOD Leu6 ThrIII, hSOD Leu6 GlnIII, hSOD Leu6 AsnIII, hSOD Leu6 MetIII, hSOD Ile6 GlyIII, hSOD Ile6 AlaIII, hSOD Ile6 ValIII, hSOD Ile6 LeuIII, hSOD Ile6 SerIII, hSOD Ile6 ThrIII, hSOD Ile6 GlnIII, hSOD Ile6 MetIII, hSOD Ser6 GlyIII, hSOD Ser6 AlaIII, hSOD Ser6 ValIII, hSOD Ser6 LeuIII, hSOD Ser6 IleIII, hSOD Ser6 ThrIII, hSOD Ser6 GlnIII, hSOD Ser6 AsnIII, hSOD Ser6 MetIII, hSOD Thr6 GlyIII, hSOD Thr6 AlaIII, hSOD Thr6 LeuIII, hSOD Thr6 IleIII, hSOD Thr6 GlnIII, hSOD Thr6 AsnIII, hSOD Thr6 MetIII, hSOD Glu6 GlyIII, hSOD Gln6 AlaIII, hSOD Gln6 ValIII, hSOD Glu6 LeuIII, hSOD Gln6 SerIII, hSOD Glu6 ThrIII, hSOD Glu6 AsnIII, hSOD Glu6 MetIII, hSOD Asn6 GlyIII, hSOD Asn6 AlaIII, hSOD Asn6 ValIII, hSOD Asn6 SerIII, hSOD Asn6 ThrIII, hSOD Asn6 MetIII, hSOD Met6 GlyIII, hSOD Met6 AlaIII, hSOD Met6 ValIII, hSOD Met6 SerIII, hSOD Met6 ThrIII, hSOD Met6 GluIII, and hSOD Met6 AsnIII.

The N-terminus of the mutein may be acetylated (as in native hSOD) or lack acetylation depending upon the organism in which the mutein is produced. Bacterially produced mutein will lack such acetylation whereas mutein produced in yeast using the procedures described in EPA 84III416.8 are so acetylated. Muteins having such acetylation are preferred. Similarly, the mutein may be glycosylated or unglycosylated depending upon the organism and signaling sequence with which it is produced.

Genes encoding the muteins of the invention may be made via oligonucleotide synthesis and ligation, site directed mutagenesis of the DNA sequence shown in Figure I and/or by insertion of synthetic DNA fragments that encode the desired amino acid substitutions into a DNA sequence encoding native hSOD. Site directed mutagenesis techniques are well known in the art. See for instance, Smith and Gilliam in Genetic Engineering Principles and Methods, Plenum Press (I98I) 3:I-32; Zoller and Smith, Nucleic Acids Res (I982) I0:6487-6500; and Brake et al, Proc Natl Acad Sci USA (I984) 8I:4642-4646. The mutant genes may be inserted into suitable prokaryotic or eukaryotic replicons (a genetic element such as a plasmid, a chromosome, or a virus that behaves as an autonomous unit of polynucleotide replication within a cell), the resulting expression vectors incorporated into suitable host organisms or cells, the recombinant organism or cell grown under conditions that result in expression of the mutant gene, and the resulting mutein isolated from the host or, if secreted, from the growth medium using the same techniques as are described in EPA 84III416.8 to produce recombinant hSOD. The disclosure of that EPA is incorporated herein by reference.

In creating an expression vector, the mutant sequence is located in the vector with the appropriate control DNA sequences, which include a promoter, a ribosomal binding site, and transcriptional and translational stop codons. The positioning and orientation of the coding sequence with respect to the control sequences is such that the coding sequence is transcribed under the "control" of the control sequences: i.e., the promoter will control the transcription of the mRNA derived from the coding sequence, the ribosomes will bind at the ribosomal binding site to begin the translational process, and the stop codon used to terminate translation will be upstream from the transcriptional termination codon. In addition to control sequence, it may be desirable to

add regulatory sequences which allow for regulation of the expression of the mutant hSOD gene r lative to the growth of the host cell.

The hSOD muteins of the invention may be used for the same purposes as hSOD. Because of their better thermostability, the muteins are more readily formulated with materials that requir the us of elevated temperatures for blending or processing. The muteins may be used in human or veterinary medicine t tr at (i.e. cure, alleviate or prevent) a variety of conditions. They are useful as antiinflammatory agents, chemopreventive agents to prevent oncogenesis and tumor promotion, protective agents to reduce cytotoxic and cardiotoxic effects of anticancer drugs or protect ischemic tissue. Like native hSOD, the muteins catalyze the reduction of superoxide radicals to hydroperoxide and molecular oxygen and may thus be used to reduce perfusion injury following ischemia, prolong the viability of excised isolated organ transplants, reduce injury on reperfusion following organ transplant or spinal cord ischemia, reduce cardiac infarct size, reduce spinal cord injury and treat bronchial pulmonary dysplasia.

For medical applications the mutein may be administered orally or parenterally to individuals in various dosage forms such as tablets, capsules, and injectables. When used to treat tissues in vitro the mutein will be added to the perfusion or culture medium. The mutein may be administered neat or admixed in effective amounts with pharmaceutically acceptable solid, semisolid or liquid vehicles such as albumins, globulins, dextran, Ficoll polymers, sugars, starches, and liposomes. Preferably the hSOD mutein is conveniently stored lyophilized with sugar, usually sucrose, usually in a ratio of 1:2 w/w. The lyophilized enzyme is conveniently reconstituted in a suitable diluent for the particular application. For example, to treat inflammatory joint disease the hSOD mutein may be reconstituted in physiologic saline in a volume convenient for intraarticular administration.

The dose of hSOD mutein administered to an individual will depend upon the nature of the individual being treated, the mode of treatment and the condition being treated. In general the amount administered must be sufficient to provide an enzymatically effective amount of the mutein at the desired site of treatment. In this regard when the mutein is administered systemically, larger doses will typically be required than when the mutein is administered locally at the site that requires treatment. By way of example, human patients having inflammatory joint disease are treated by a weekly intraarticular injection into a joint afflicted with the disease of a solution having hSOD mutein in a suitable diluent in an amount effective to reduce inflammation, usually 1 to 10 mg, more usually 2 to 6 mg. The injections are given weekly for a period of time sufficient to reduce inflammation, usually for 2 to 8 weeks, more usually for 4 to 6 weeks. Because the articular capsule limits leakage of the high molecular weight compound each afflicted joint should be treated with the required dosage. When used to minimize postischemic tissue damage the human patient is administered 10 mg to 1,000 mg, more usually 50 mg to 500 mg of hSOD mutein in a suitable diluent during the ischemic reaction. When the patient suffers ischemia due to a disease the solution is administered intraveneously or intraarterially as a bolus dosage or a continuous infusion. In such situations the hSOD mutein may be administered in conjunction with fibrinolytic agents such as urokinase, streptokinase or tissue plasminogen activator (TPA). When ischemic damage is due to a surgical procedure, hSOD mutein is administered during surgery. This application finds particular use in organ transplant surgery where hSOD is preferably administered prior to reirrigation of the organ and is also useful in any other surgery where bloodflow to an organ is interrupted, such as open heart surgery.

The hSOD muteins may also be used in cosmetic compositions for skin or hair care as a protective agent for keratinic substances or to prevent oxidative degradation of components of such compositions. For instance they may be added to such formulations to maintain or improve skin or hair qualities such as softness, flexibility or elasticity or prevent oxidation of oxidizable or self-oxidizable substances such as dyes used in cosmetic preparations. The form of the mutein-containing cosmetic formulation may be solid (e.g. cleansing bars), semisolid (e.g. creams, gels, ointments) or liquid (e.g. sprays, lotions, shampoos). The amount of hSOD mutein in such formulations will normally be in the range of about 0.01% to 5% by weight, more usually 0.05% to 1% by weight. In addition to the hSOD mutein, the cosmetic preparation may contain: fatty carrier materials such as natural oils (e.g. olive oil, avocado oil, and the like), fatty acid esters such as stearin, glyceryl monostearate, ethyl palmitate, cetyl myristate, isopropyl oleate and the like, alcohols such as cetyl alcohol or polyoxyethylenated fatty alcohols, waxes such as beeswax or synthetic waxes; dyes, perfumes, surfactants, preservatives, thickeners, or other additives conventionally included in cosmetics.

They may also be added to foods as preservatives to prevent oxidative degradation of food components.

The following examples further illustrate the invention. These examples are not intended to limit the invention in any manner.

Construction of Yeast Plasmid pYSODA Encoding hSOD Ala6 Expression

The construction of plasmid pYSODA is depicted in Figure 2.

A DNA adaptor fragment (designated B in Figure 2) having the sequence shown below was synthesized.

```
NcoI    1  2  3  4  5  6                                        TaqI
   MetAlaThrLysAlaValAlaValLeuLysGlyAspGlyProValGlnGlyIleIleAsnPh
 CATGGCTACAAAGGCTGTTGCTGTTTTGAAGGGTGACGGGCCCGTTCAAGGTATTATTAACTT
    CGATGTTTCCGACAACGACAAAACTTCCCACTGCCCGGGCAAGTTCCATAATAATTGAAGC
```

The fragment was phosphorylated on the coding (upper) strand only and was designed to encode the N-terminal sequence of hSOD with the codon for amino acid 6 altered to encode an alanine rather than a cysteine and have an NcoI site at its 5′ end and a TaqI site at its 3′ end.

Plasmid pASIIr was linearized with NcoI and ligated to adaptor B. Plasmid pASIIr consists of the expression cassette of plasmid pASII (the hSOD gene fused to the N-terminus of the human proinsulin gene with a methionine codon at the junction of the two genes under the regulation of a hybrid inducible S. cerevisae alcohol dehydrogenase 2-glyceraldehyde phosphate dehydrogenase (ADH2-GAP) promoter and the GAP terminator) in the S.R. 322 vector. Details of the composition and construction of plasmid pASII are described in commonly owned European Patent Application Serial No. 86104066.5, the disclosure of which as it relates to pASII is incorporated herein by reference. (The plasmid is designated pYASII in Serial No. 86104066.5.) The S.R. 322 vector is described in J Biol Chem (1985) 260:4384-4389. The ligation product was cut with SalI and the resulting large fragment was gel purified.

Plasmid pSII/8 was cut with SalI and TaqI and a 660 bp TaqI - SalI fragment consisting of a portion of the hSOD gene fused to the N-terminus of the human proinsulin gene was gel isolated from the digest. Plasmid pSII/8 contains the hSOD gene fused to the N-terminus of the human proinsulin gene under the regulation of the GAP promoter and terminator. Details of its composition and construction are described in commonly owned European Patent Application Serial No. 86104066.5, the disclosure of which, as it relates to pSII/8, is incorporated herein by reference. (The plasmid is designated pYSII in Serial No. 86104066.5.)

The 660 bp TaqI - SalI fragment was ligated to the linear pASIIr - adaptor B construct to produce plasmid pSI6. That plasmid was cut with NcoI and StuI and a 126 bp fragment gel isolated from the digest.

The plasmid pGAPSOD was cut with NcoI and StuI and vector DNA was gel purified. pGAPSOD is a yeast promoter vector containing the hSOD gene under the regulation of the GAP promoter and terminator. Details of its composition and construction are described in commonly owned European Patent Application Serial No. 84111416.8, the disclosure of which, as it relates to this plasmid, is incorporated herein by reference. (The plasmid is designated pPGAPSOD in Serial No. 84111416.8.) The 126 bp NcoI - StuI fragment from pSI6 was ligated with the vector DNA to form plasmid pSODA. pSODA was then cut with BamHI and a BamHI expression cassette having the hSOD gene with an Ala codon at amino acid 6 flanked by GAP promoter and terminator sequences was isolated. This expression cassette was then cloned in the expression orientation in plasmid pCI/I, which had been digested with BamHI and phosphatased, to produce pYSODA. Plasmid pCI/I is a derivative of pJDB219 (Beggs, Nature (1978) 275:104) in which the region corresponding to bacterial plasmid pMB9 in pJDB219 is replaced by pBR322.

Construction of Yeast Plasmid pYSODS encoding hSOD SerIII Expression

The mutagenesis of the hSOD CysIII codon to a ser-encoding triplet was accomplished using MI3 site directed mutagenesis. The construction of the mutant MI3 plasmid and related plasmids involved in the construction of pYSODS are depicted in Figures 3 and 4 and described below.

Plasmid pSODX8 (described in Nucleic Acids Res (1985) 13:2017-2034) was cut with StuI and SalI. An approximately 400 bp fragment was gel isolated and partially digested with Sau3A and a 327 bp hSOD fragment was recovered. Plasmid pSODXI6 (described in Nucl Acids Res (1985) 13:2017-2033) was cut with StuI and BamHI and the large vector fragment was gel isolated from the digest. This vector fragment was ligated to the 327 bp hSOD fragment to produce plasmid pSODCFI.

Plasmid pSI8, an hSOD-proinsulin fusion expression plasmid derived from pYASII (see above) and consisting of the expression cassette of pYASII in the S.R. 322 vector was cut with NcoI and StuI and a 124 bp fragment was gel isolated from the digest. That fragment was ligated with a 330 bp fragment isolated from pSODCFI digested with StuI and BamHI to yield a 454 bp NcoI - BamHI fragment. That fragment was amplified by cloning in plasmid pHBS6 (described in European Patent Application Serial No. 85105405.6, the disclosure of which, as it relates to pHBS6, is incorporated herein by reference).

The phage vector MI3rp8 was cut with NcoI and BamHI and the resulting vector fragment ligated to the 454 bp hSOD fragment to produce the vector MI3rp8SOD-I. Single-stranded DNA was prepared from this vector to serve as a template for site directed in vitro mutagenesis using the following synthetic oligonucleotide primer
5′-GCCAATGATGGAATGGTCTCCTTGAGAGTGATATCACAGAATC-3′.
The mutagenesis was carried out according to the procedure of Brake et al, supra. The mutant RF MI3 clone containing the hSOD CysIII gene (designated MI3rp8SODCIIIS) was identified by screening with a ^{32}P-labeled probe having the sequence
5′-TTGAGAGTGATATCACAGAATC-3′.
The mutant MI3 clone MI3rp8SODCIIIS was cut with StuI and BamHI and a 330 bp StuI - BamHI fragment isolated from the digest. That fragment was ligated to a vector fragment prepared by digesting pSODCFI with StuI and BamHI. The resulting plasmid, pSODXCS, was digested with BamHI and a synthetic adaptor

(designated (C) in Figure 5) having the sequence given below was ligat d to the vector

```
BamHI                                                    SalI
 yIleAlaGlnOC
GATCGCCCAATAAACATTCCCGGGGATGTAGTCTGAGGCCCCTTAACTCATCTGTTATCCTGCTAG
    CGGGTTATTTGTAAGGGCCCCTACATCAGACTCCGGGGAATTGAGTAGACAATAGGACGATCAGCT
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The ligation product was then cut with StuI and a 260 bp StuI - SalI fragment was gel isolated. This fragment was then ligated to vector DNA prepared by digesting pGAPSOD with StuI and SalI to produce the plasmid pSODS. pSODS was then cut with BamHI and a fragment consisting of the hSOD CysIII gene flanked by GAP promoter and terminator sequences was isolated from the digest. That fragment was ligated into the vector pCI/I that had been cut with BamHI and phosphatased to produce the plasmid pYSODS.

Construction of Yeast Expression Plasmid pYSODAS for Expression of hSOD Ala6 SerIII

The construction of pYSODAS is diagrammed in Figure 6.

The above-described 260 bp fragment consisting of the BamHI - StuI fragment from pSODXCS with the adaptor (C) ligated to the BamHI end thereof was cloned into pSODA cut with StuI and SalI. The resulting plasmid pSODAS was cut with BamHI to produce a fragment consisting of the gene for hSOD Ala 6 SerIII flanked by GAP promoter and terminator sequences. That fragment was inserted into BamHI cut pCI/I to produce pYSODAS.

Preparation of Recombinant Yeast Strains

Plasmids pYSODA, pYSODS, and pYSODAS were transformed into yeast strain 2150-2-3 as described in PNAS USA (1978) 75:1929-1933 to produce recombinant strains that produce hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII.

Yeast Growth and hSOD Mutein Purification

Ten liter cultures of yeast strains producing hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII were grown by inoculating 10 L of YEPD medium containing 3 mM $CuSO_4$ with 500 ml of starter culture in minimal glucose medium lacking leucine (Sherman, F., Fink, G.R., & Hicks, J.B., Methods in Yeast Genetics, (1982), Cold Spring Harbor, New York) supplemented with 50 µM $CuSO_4$ and grown to $OD_{650} \sim 20$.

Yeast pastes (100-250 g wet weight) were lysed in 20 mM Tris-HCl, pH 8 (buffer), using a Dynomill Bead Disrupter. The lysate was centrifuged, and the pellet washed twice with buffer. The washes wre pooled with the original supernatant. After centrifugation, approximately l liter of extract was obtained, adjusted to pH 8 with NaOH, and stored at -20°C. The extract was thawed and heated for 2 hr at 65°C. After centrifugation to remove the precipitated protein and debris, 850 ml of clarified extract was obtained. Buffer was added to final volume of approximately 8.5 liters, resulting in a conductivity of 1.2 mmho. This was loaded onto a 400 ml column of DEAE-Sepharose. The column was washed with the same buffer, and eluted with a gradient up to 0.15 M NaCl in buffer. The peak was collected in 3 pools: A, which contains the bulk of the material from the center of the peak; B, containing the early side fractions; and C, containing the later side fractions. The pools were sterile filtered and aliquots stored at 4°C and at -20°C. Photographs of the gels of these fractions are shown in Figure 7.

The procedure resulted in a 2.5-fold purification, yielding protein at least 95% pure (see figure 7). Due to the high expression level (approximately 40% of the total protein), 2.5-fold purification is all that is possible. The overall yield is ~ 60%; the heating step resulted in an 83% yield, and the column ~ 70%. The heating step is valuable in that it purifies the hSOD mutein, and also clarifies the extract which otherwise is turbid with debris that is difficult to remove. The DEAE-Sepharose column removes the remaining contaminating proteins and other nonproteinaceous contaminants such as lipid, carbohydrate, and nucleic acid.

The purified hSOD mutein was compared to that present in the crude extract to ensure that not only was the activity present, but that the protein itself was unmodified. This is particularly important because of reactions that could occur during the heat step. By native gel electrophoresis in agarose gels, it is usually possible to separate 5 charged isomers to hSOD. These are called forms +2, +1, 0, -1 and -2. In fact, we found by native polyacrylamide gel and agarose gel electrophoresis, the hSOD Ala6 SerIII is apparently unaltered after heating. As seen in Figure 8A, when extract is heated for 2 hr at 65 or 70°C, the pattern of bands seen after agarose gel electrophoresis is only slightly changed. This is a loss of some minor, very rapidly migrating species, but otherwise there is no change.

The specific activity of hSOD Ala6 SerIII (3400 U/mg) is approximately the same as wild type hSOD (3500 U/mg) as assayed by the pyrogallol method, Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur J Biochem (1974) 47:469-474).

Thermal Stability of hSOD, hSOD Ala6, hSOD SerIII and hSOD Ala6 SerIII

Analysis of the thermostability of the hSOD muteins made in yeast was performed by heating crude extracts at 80°C for 10 min and 25 min and comparing the superoxide dismutase activity of the heated SODs with that of the unheated control on an SOD activity gel as shown in Figure 9 (Beauchamp, C. and Fridovich, I., Analyt

Biochem (1971) 44:276-287). The results depicted in Figure 9 indicate that hSOD Ala6 Ser111 is detectably more thermostable that hSOD and hSOD Ala6 and hSOD Ser111. hSOD Ala6 and hSOD Ser111 also appear to b more thermostable than wild type hSOD.

Thermal Stability of hSOD Ala6 Ser111

A. At pH 4.0. The thermal stabilities of purified recombinant hSOD, bovine SOD and hSOD Ala6 Ser111 were determined in 20 mM sodium acetate, pH 4, at a protein concentration of 20 µg/ml. After heating, the SOD activity was measured by the pyrogallol method. Figure 10 shows the melting curve seen after 1 hr of heating at temperatures from 50° to 70°C. Note that hSOD Ala6 Ser111 exhibits the most resistance to thermal denaturation under these conditions, followed by bovine SOD, and then by hSOD. After 1 hr at 60°C, hSOD Ala6 Ser111 has retained 86% of its activity, while the bovine SOD and hSOD have retained 68% and 48% activity, respectively.

Figure 11 shows the rate of denaturation at 60°C. Here the differences after 1 hr are less than the previous experiment (69% vs. 62% vs. 56%), but the relative stabilities are consistent with the earlier result.

The increased stability of the hSOD Ala6 Ser111 is especially evident after heating at 70°C (data not shown). Here, after 30 min, the hSOD Ala6 Ser111 retained 29% of its activity vs. ~ 10% for the other SODs. And even after 90 min at 70°C, the hSOD Ala6 Ser111 still had 25% of its original activity while the others had less than 10% activity.

B. At pH 7.8. The experimental conditions were identical to those at pH 4, exzcept that the buffer was 0.1 M sodium phosphate, pH 7.8. Figure 12 shows the rates of inactivation at 70°C. Here, the differences in stability are dramatic; the hSOD Ala6 Ser111 is most stable, followed by bovine SOD and then hSOD. These are the same relative stabilities seen at pH 4, but at pH 7.8 the hSOD Ala6 Ser111 now has a 10-fold longer half-life at 70°C than hSOD (175 vs. 16 min). It is also more than 2-fold more stable than bovine SOD (175 vs. 84 min).

Modifications of the above described modes for carrying out the invention that are obvious to those of ordinary skill in the fields of protein chemistry, genetic engineering, medicine and related fields are intended to be within the scope of the following claims.

Claims

1. A mutein of human Cu/Zn superoxide dismutase in which at least one of the cysteine residues at positions 6 and 111 is replaced with an uncharged amino acid.
2. The mutein of claim 1 wherein said uncharged amino acid is acyclic.
3. The mutein of claim 2 wherein the side chain of the uncharged acyclic amino acid is hydrogen, an aliphatic side chain or a hydroxylic side chain.
4. The mutein of claim 1, 2 or 3 wherein the N-terminus of the mutein is acetylated.
5. The mutein of claim 1, 2, 3, or 4 wherrein only one of said cysteine residues is replaced with an uncharged amino acid.
6. The mutein of claim 5 wherein the one of said cysteine residues is at position 6.
7. The mutein of claim 5 wherein the one of said cysteine residues is at position 111.
8. The mutein of claim 1, 4, 5, 6, or 7 wherein the uncharged amino acid is glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, or methionine.
9. The mutein of claim 1, 2, 3, or 4 wherein both of the cysteine residues are replaced with an uncharged amino acid.
10. The mutein of claim 9 wherein the uncharged amino acid is glycine, valine, alanine, leucine, isoleucine, serine, threonine, asparagine, glutamine, or methionine.
11. The mutein of claim 9 wherein the cysteine residue at position 6 is replaced with alanine and the cysteine residue at position 111 is replaced with serine.
12. A method of enhancing the thermostability of human Cu/Zn superoxide dismutase comprising replacing at least one of the cysteine residues at positions 6 and 111 with an uncharged amino aid.
13. The method of claim 12 wherein only the cysteine residue at position 6 is replaced and the uncharged amino acid is serine.
14. The method of claim 12 wherein only the cysteine residue at position 111 is replaced and the uncharged amino acid is serine.
15. The method of claim 12 wherein the cysteine residue at position 6 is replaced with alanine and the cysteine residue at position 111 is replaced with serine.
16. A pharmaceutical composition comprising an enzymatically effective amount of the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 admixed with a pharmaceutically acceptable carrier.
17. A cosmetic composition comprising an enzymatically effective amount of the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 admixed with a carrier.
18. DNA encoding the mutein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11.
19. An expression vector for expressing the DNA of claim 18 in an organism comprising the DNA of claim 18 operably connected to DNA that enables the expression of the DNA of claim 18 in the organism.
20. A host organism containing the vector of claim 19 which permits the expression of said mutein-encoding DNA.

```
CTGCAGCGTCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGTGGCCTAGC


                1                                          10
          Met Ala Thr Lys Ala Val Cys Val Leu Lys Gly Asp Gly
GAGTT     ATG GCG ACG AAG GCC GTG TGC GTG CTG AAG GGC GAC GGC

                                   20
Pro Val Gln Gly Ile Ile Asn Phe Glu Gln Lys Glu Ser Asn Gly
CCA GTG CAG GGC ATC ATC AAT TTC GAG CAG AAG GAA AGT AAT GGA

          30                                          40
Pro Val Lys Val Trp Gly Ser Ile Lys Gly Leu Thr Glu Gly Leu
CCA GTG AAG GTG TGG GGA AGC ATT AAA GGA CTG ACT GAA GGC CTG

                                   50
His Gly Phe His Val His Glu Phe Gly Asp Asn Thr Ala Gly Cys
CAT GGA TTC CAT GTT CAT GAG TTT GGA GAT AAT ACA GCA GGC TGT

          60                                          70
Thr Ser Ala Gly Pro His Phe Asn Pro Leu Ser Arg Lys His Gly
ACC AGT GCA GGT CCT CAC TTT AAT CCT CTA TCC AGA AAA CAC GGT

                                   80
Gly Pro Lys Asp Glu Glu Arg His Val Gly Asp Leu Gly Asn Val
GGG CCA AAG GAT GAA GAG AGG CAT GTT GGA GAC TTG GGC AAT GTG

          90                                         100
Thr Ala Asp Lys Asp Gly Val Ala Asp Val Ser Ile Glu Asp Ser
ACT GCT GAC AAA GAT GGT GTG GCC GAT GTG TCT ATT GAA GAT TCT

                                  110
Val Ile Ser Leu Ser Gly Asp His Cys Ile Ile Gly Arg Thr Leu
GTG ATC TCA CTC TCA GGA GAC CAT TGC ATC ATT GGC CGC ACA CTG

         120                                         130
Val Val His Glu Lys Ala Asp Asp Leu Gly Lys Gly Gly Asn Glu
GTG GTC CAT GAA AAA GCA GAT GAC TTG GGC AAA GGT GGA AAT GAA

                                  140
Glu Ser Thr Lys Thr Gly Asn Ala Gly Ser Arg Leu Ala Cys Gly
GAA AGT ACA AAG ACA GGA AAC GCT GGA AGT CGT TTG GCT TGT GGT

         150          153
Val Ile Gly Ile Ala Gln OC
GTA ATT GGG ATC GCC CAA TAA
```

FIG. 1

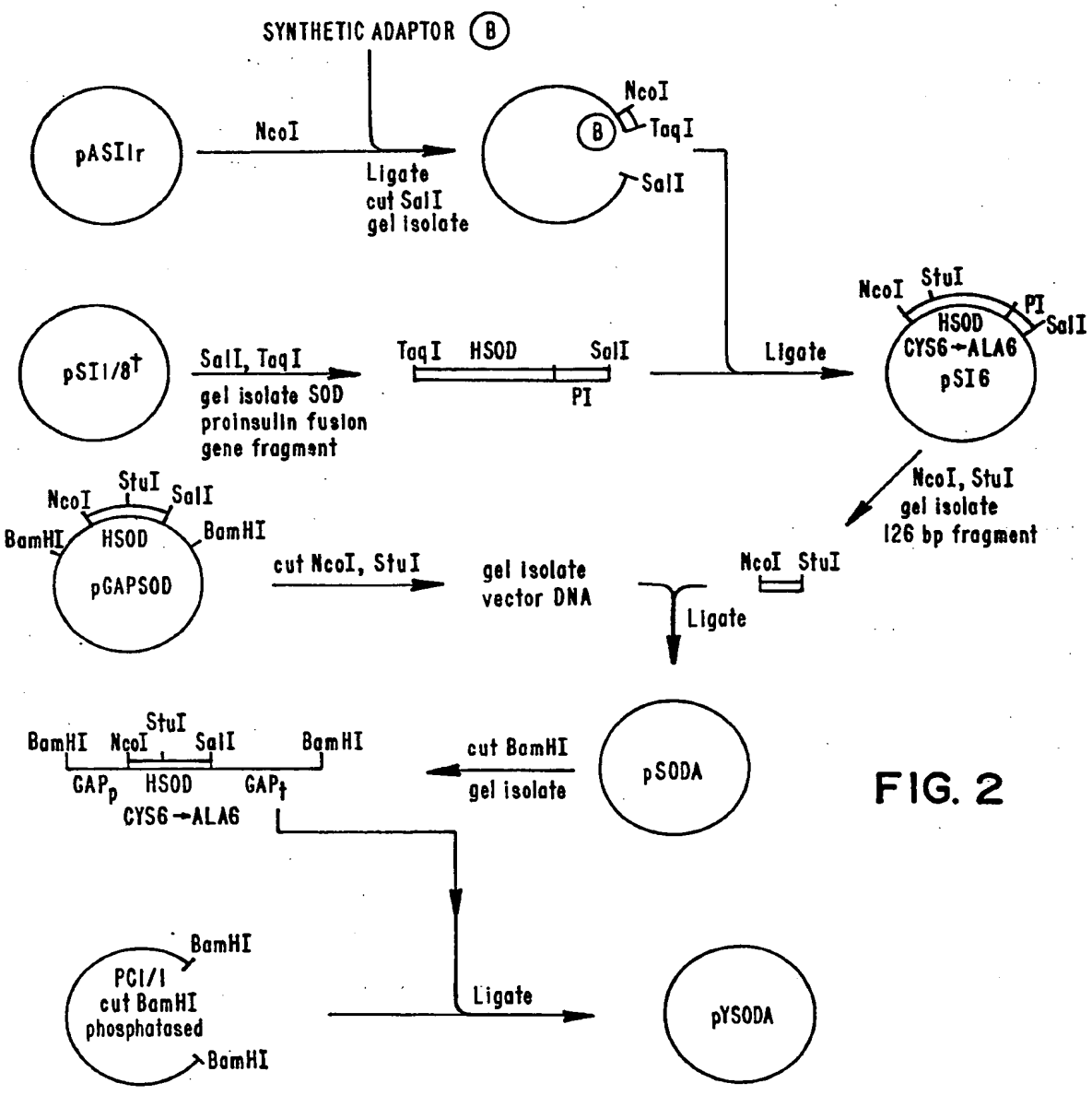

SYNTHETIC ADAPTOR B
pASIIr
NcoI
Ligate
cut SalI
gel isolate
NcoI
B
TaqI
SalI
pSI1/8†
SalI, TaqI
gel isolate SOD
proinsulin fusion
gene fragment
TaqI HSOD SalI
PI
Ligate
NcoI StuI
PI
HSOD
CYS6→ALA6
SalI
pSI6
NcoI, StuI
gel isolate
126 bp fragment
NcoI StuI SalI
BamHI
HSOD
BamHI
pGAPSOD
cut NcoI, StuI
gel isolate
vector DNA
NcoI StuI
Ligate
BamHI NcoI StuI SalI BamHI
GAPp HSOD GAPt
CYS6→ALA6
cut BamHI
gel isolate
pSODA
BamHI
PCI/1
cut BamHI
phosphatased
BamHI
Ligate
pYSODA

FIG. 2

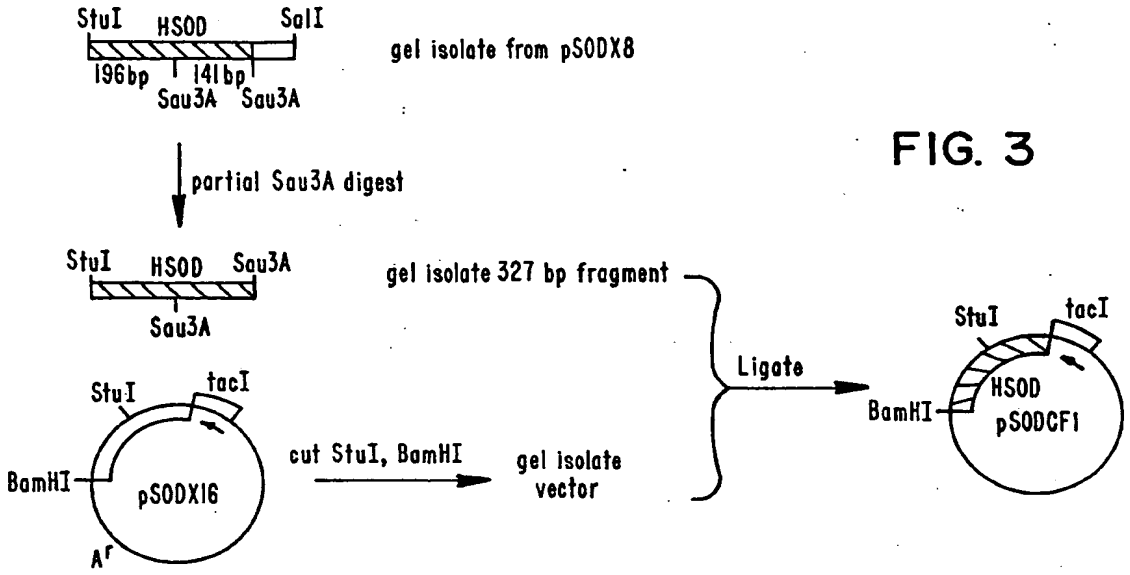

StuI
HSOD
SalI
196bp
141bp
Sau3A
Sau3A
gel isolate from pSODX8
partial Sau3A digest
StuI
HSOD
Sau3A
Sau3A
gel isolate 327 bp fragment
Ligate
StuI
tacI
HSOD
BamHI
pSODCF1
StuI
tacI
BamHI
pSODX16
A^r
cut StuI, BamHI
gel isolate
vector

FIG. 3

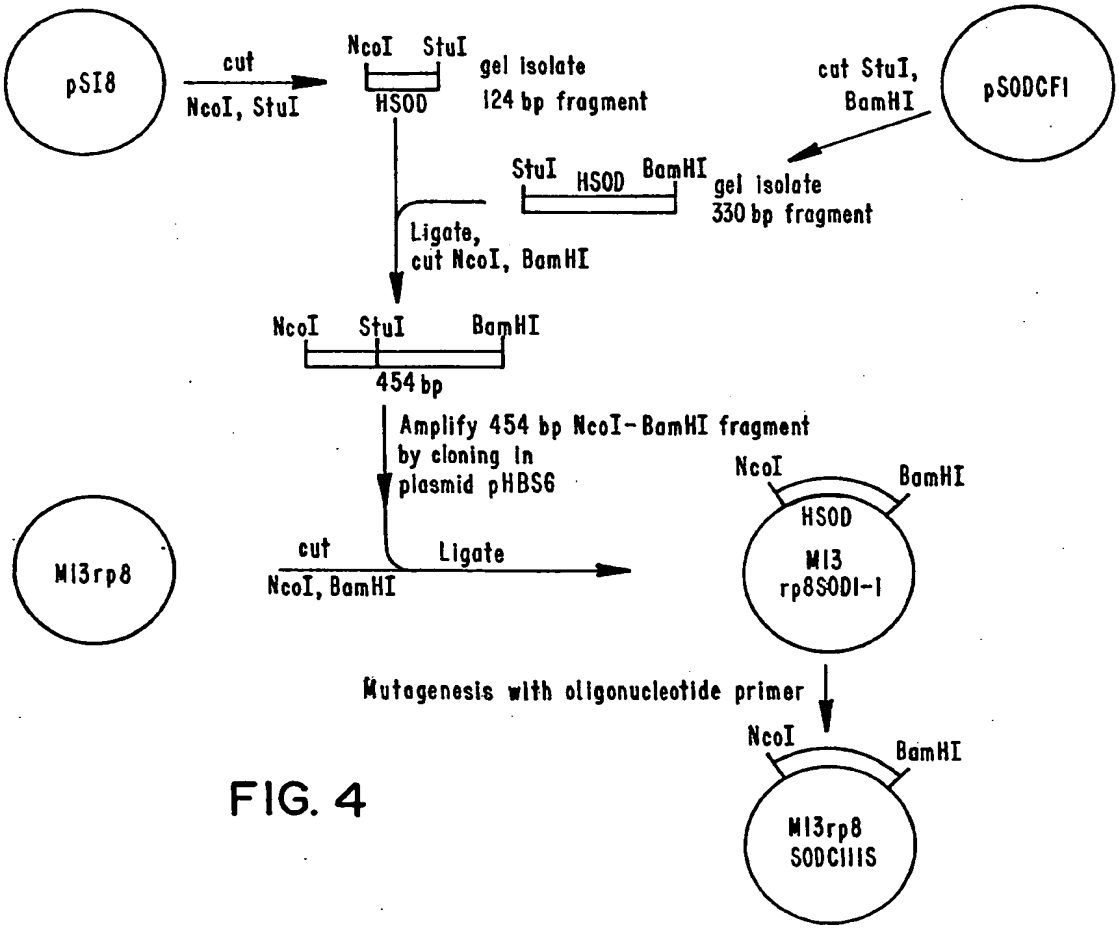

pSI8
cut
NcoI, StuI
NcoI
StuI
HSOD
gel isolate
124 bp fragment
cut StuI,
BamHI
pSODCF1
StuI
HSOD
BamHI
gel isolate
330 bp fragment
Ligate,
cut NcoI, BamHI
NcoI
StuI
BamHI
454 bp
Amplify 454 bp NcoI-BamHI fragment
by cloning in
plasmid pHBS6
M13rp8
cut
NcoI, BamHI
Ligate
NcoI
BamHI
HSOD
M13
rp8SODI-1
Mutagenesis with oligonucleotide primer
NcoI
BamHI
M13rp8
SODCIIIS

FIG. 4

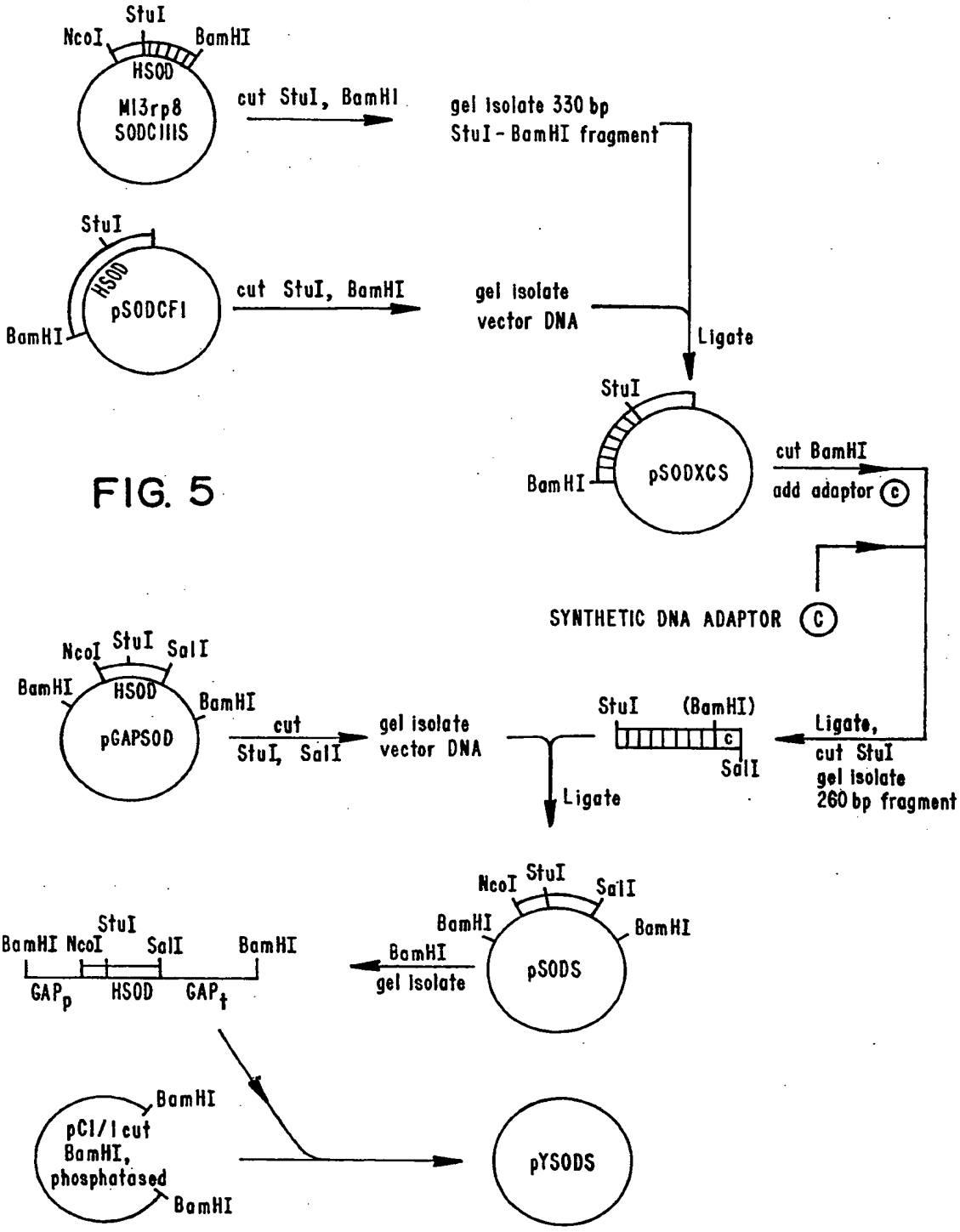

StuI
NcoI
BamHI
HSOD
M13rp8
SODCIIIS
cut StuI, BamHI
gel isolate 330 bp
StuI - BamHI fragment
StuI
HSOD
pSODCF1
BamHI
cut StuI, BamHI
gel isolate
vector DNA
Ligate
StuI
BamHI
pSODXCS
cut BamHI
add adaptor C
SYNTHETIC DNA ADAPTOR C
NcoI StuI SalI
BamHI
HSOD
BamHI
pGAPSOD
cut
StuI, SalI
gel isolate
vector DNA
StuI (BamHI)
C
SalI
Ligate,
cut StuI
gel isolate
260 bp fragment
Ligate
StuI
BamHI NcoI SalI BamHI
GAPp HSOD GAPt
NcoI StuI SalI
BamHI
BamHI
BamHI
gel isolate
pSODS
BamHI
pCI/1 cut
BamHI,
phosphatased
BamHI
pYSODS

FIG. 5

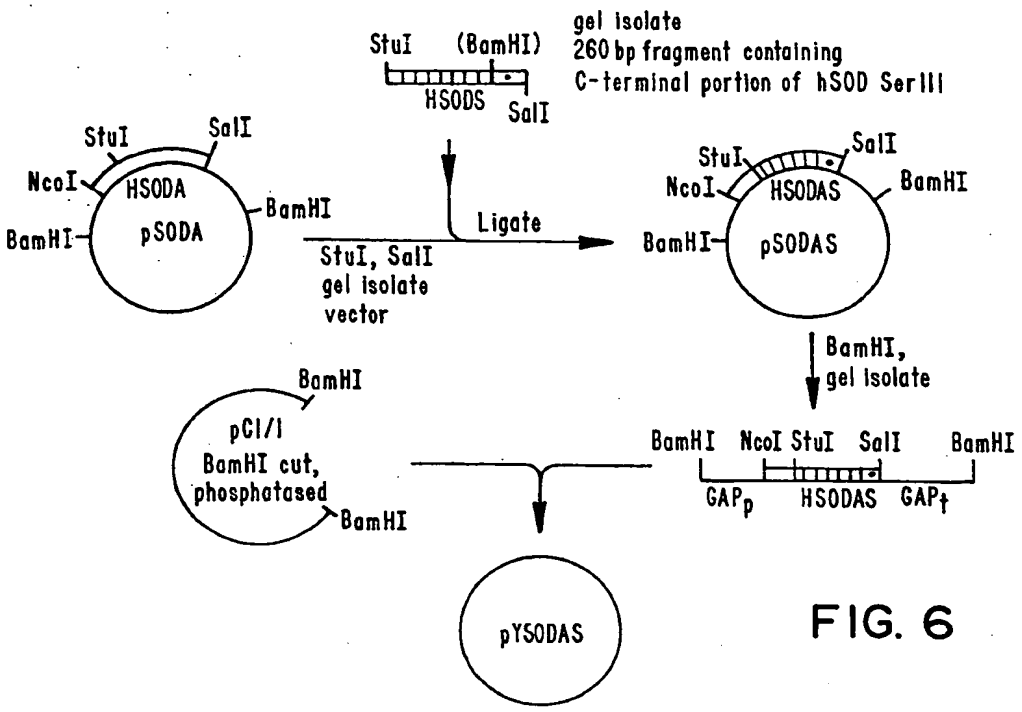

StuI
(BamHI)
HSODS
SalI
gel isolate
260 bp fragment containing
C-terminal portion of hSOD Ser III
StuI
SalI
NcoI
HSODA
BamHI
pSODA
BamHI
Ligate
StuI, SalI
gel isolate
vector
StuI
SalI
NcoI
HSODAS
BamHI
BamHI
pSODAS
BamHI,
gel isolate
BamHI
pCI/I
BamHI cut,
phosphatased
BamHI
BamHI NcoI StuI SalI BamHI
GAP$_p$ HSODAS GAP$_t$
pYSODAS

FIG. 6

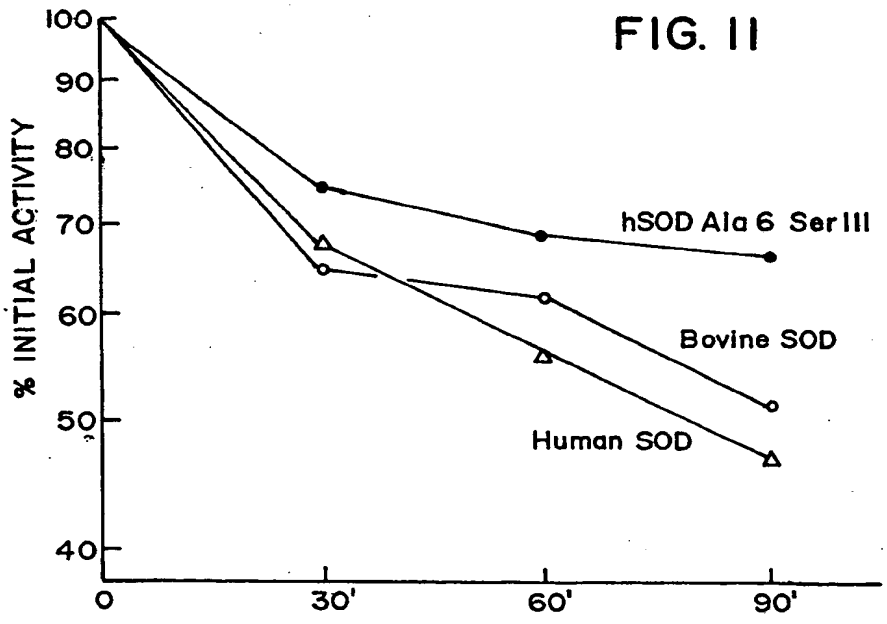

100
90
80
70
60
50
40
% INITIAL ACTIVITY
0
30'
60'
90'
hSOD Ala 6 Ser III
Bovine SOD
Human SOD

FIG. 11

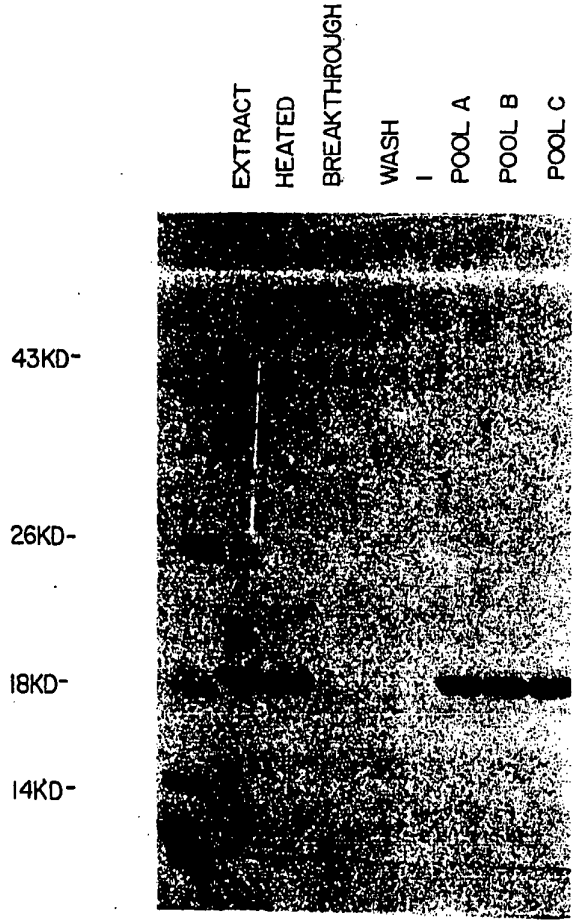


FIG. 7

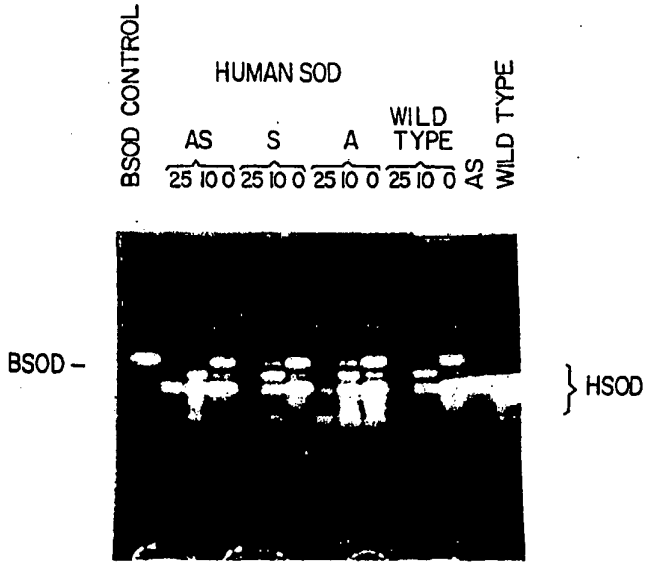


FIG. 9

0275202

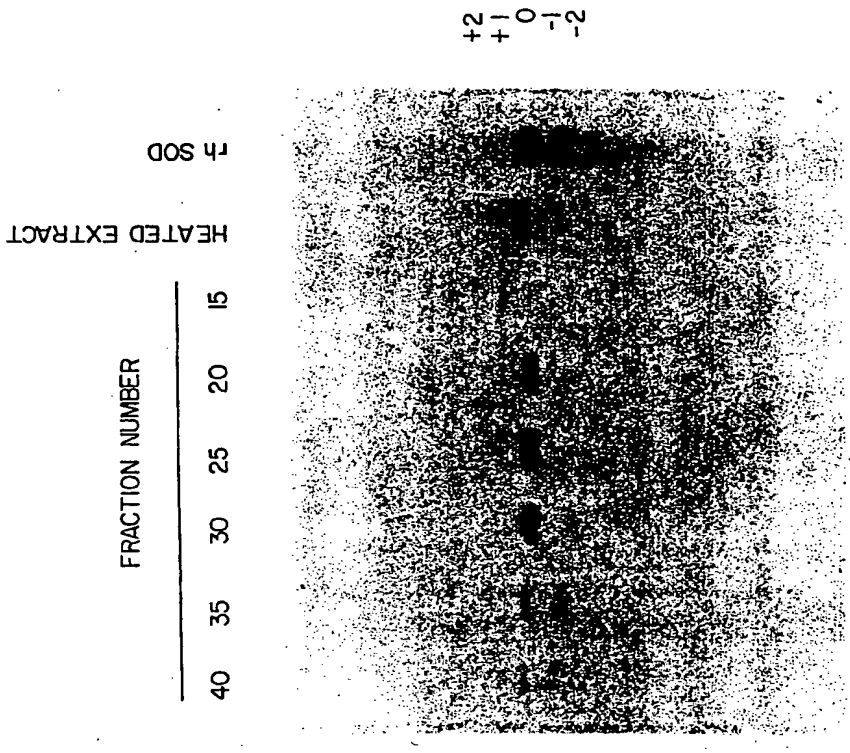

+2
+1
0
-1
-2
rh SOD
HEATED EXTRACT
FRACTION NUMBER
15
20
25
30
35
40

FIG. 8B


70°C
65°C
4°C

FIG. 8A

FIG. 12

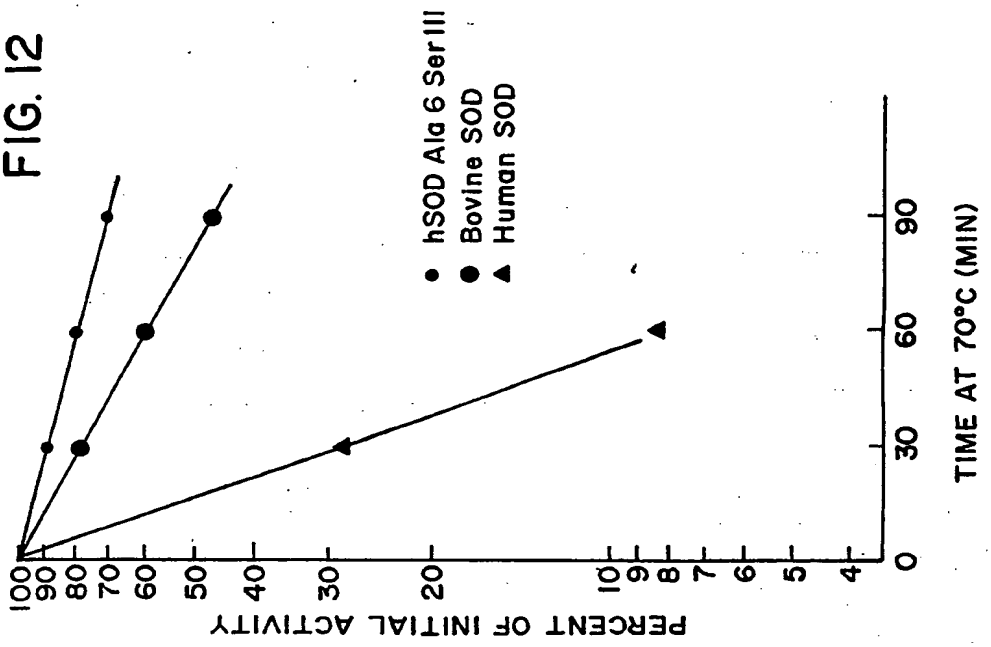
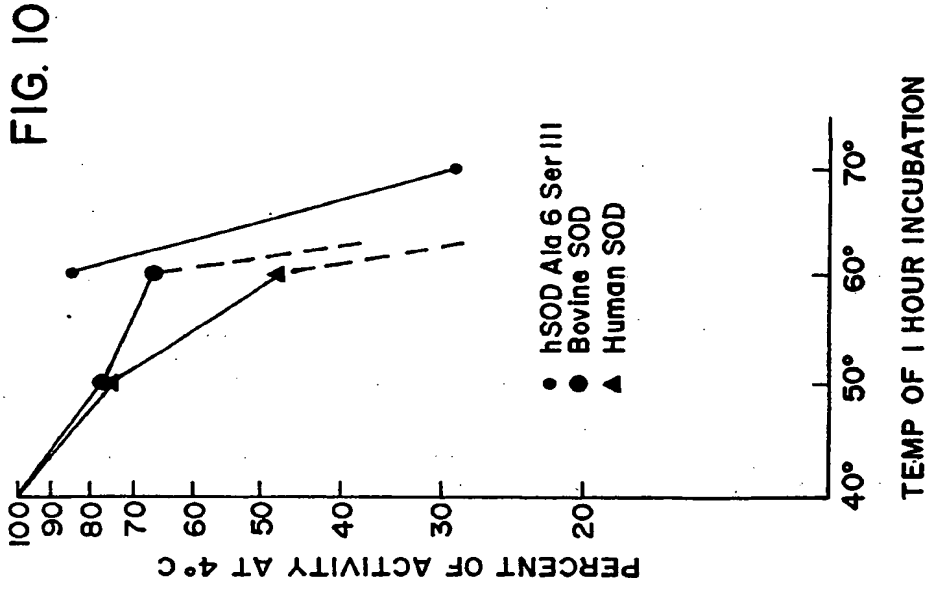

PERCENT OF INITIAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
0
30
60
90
TIME AT 70°C (MIN)
hSOD Ala 6 Ser III
Bovine SOD
Human SOD

FIG. 10


PERCENT OF ACTIVITY AT 4°C
100
90
80
70
60
50
40
30
20
40°
50°
60°
70°
TEMP OF 1 HOUR INCUBATION
hSOD Ala 6 Ser III
Bovine SOD
Human SOD